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Award Number: W81XWH-10-1-0614

TITLE: Laser Capture Microdissection Assisted Identification of Epithelial MicroRNA Expression Signatures for Prognosis of Stage I NSCLC

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REPORT DATE: October 2011

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2011	Annual	7 September 2010 – 6 September 2011

4. TITLE AND SUBTITLE

Laser Capture Microdissection Assisted Identification of Epithelial MicroRNA Expression Signatures for Prognosis of Stage I NSCLC

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-10-1-0614

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Health Research, Inc.
Roswell Park Cancer Institute Division
Buffalo, NY 14263

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The primary aim of this project is to laser microdissect stage I lung cancer samples, perform microRNA profiling of the epithelial and stromal components and develop component specific signatures of prognosis.This report summarizes the work performed on the project so far. A significant body of work has been performed to optimize the extraction of RNA from formalin fixed paraffin embedded lung cancer tissue and a working protocol established. These finding are useful to the scientific community and have been submitted for publication. Fifty seven of the proposed 77 stage I lung cancer samples have been microdissected this far into their epithelial and stromal component separated. Enough dissection has been performed to successfully acquire 400 ng of total RNA from each component.

15. SUBJECT TERMS
Lung cancer; microRNA, prognosis, biomarkers, laser microdissection

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	40	19b. TELEPHONE NUMBER *(include area code)*

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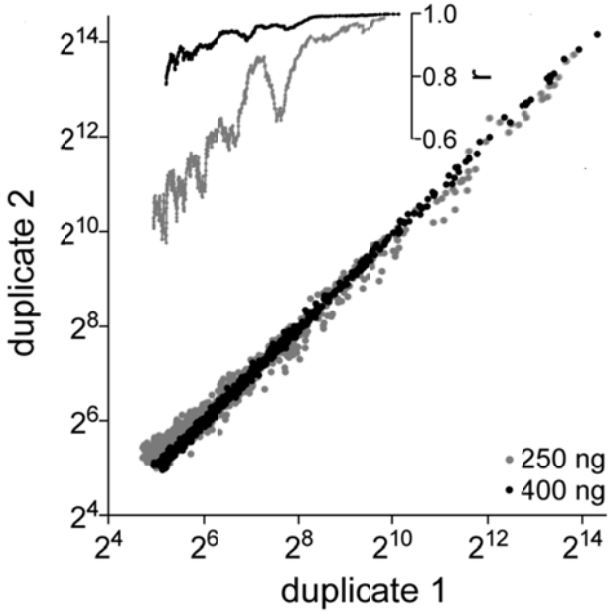
Introduction:

Even stage I lung cancer patients have an unacceptably high rate of recurrence (~35%)[1]. A prognostic assay can help identify patients for intensified treatment such as adjuvant chemotherapy. Our previous data has demonstrated the potential of microRNA profiling of whole tumors to prognosticate early non-small cell lung cancer (NSCLC)[2]. However, some of the prognostic "signal" may be masked by varying composition of whole tumors with respect to their epithelial and stromal components. In this project, we intend to perform laser capture microdissection of lung cancer specimens to separate out the epithelial and stromal components of tumors and perform microRNA profiling of these separate components to try to improve our prognostic assay and to identify specific cancer epithelial microRNA of biological import.

Progress report:

A) Optimization of LMD protocol for microRNA quantitation by microarray:

In our preliminary data submitted in the proposal, the feasibility of microRNA profiling using laser capture microdissected specimens was established using 100 ng input RNA from a few samples with quantification of the RNA being performed by absorbance at 260 nm using the Nanodrop device. However, before embarking on this major project, we sought to optimize variables that may influence miRNA quantification by microarray and to set up a feasible workflow to enable a reasonably high throughput processing of tissue. One of the first variables tested was the amount of total RNA input needed for reproducible microarray quantification. In order to do this, 250 or 400 ng of the same RNA sample obtained by LCM was labeled with the Hy3 dye and hybridized in duplicate to miRCURY^{TM} (Exiqon Inc, Denmark). With both amounts, ~56% of the 1291 microRNAs detectable by the microarrays were identified as expressed. Not surprisingly, microarray signals were stronger with higher RNA input (Figure 1). However, interduplicate correlation analyses clearly demonstrated



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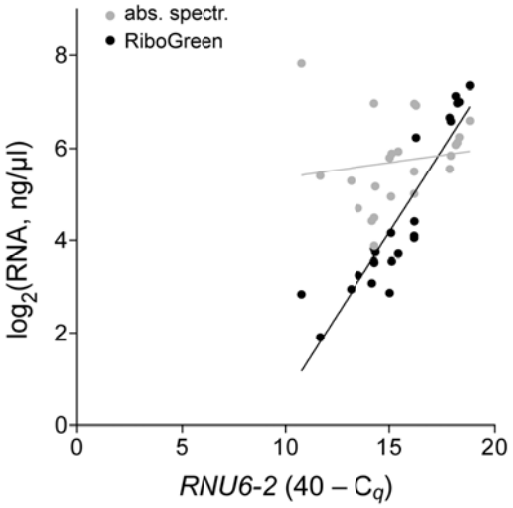
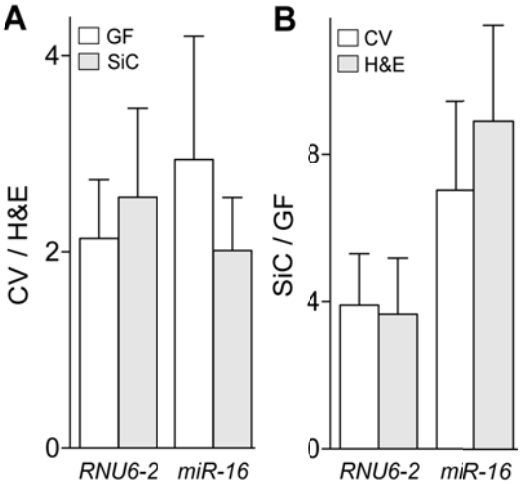
microRNA quantifications were more accurate and less noisy when 400 ng of RNA was used. As demonstrated in the inset in Figure 1, reliable quantitation of a much greater number of microRNAs are found with 400 ng than with 250 ng, a finding not apparent when only the number of microRNA considered expressed is measured. This finding was clearly important to our proposed project. While an increase in total RNA input can be simply increased by an increase in the total area of dissection performed, a fourfold increase in the area of dissection is not practical, given that, in some specimens, 6-8 hours of dissection is required to obtain 100 ng of total RNA. Therefore, we evaluated individual variables affecting RNA yield from microdissectates and in a series of experiments to boost our yield. A summary of the results from our experiments is summarized below:

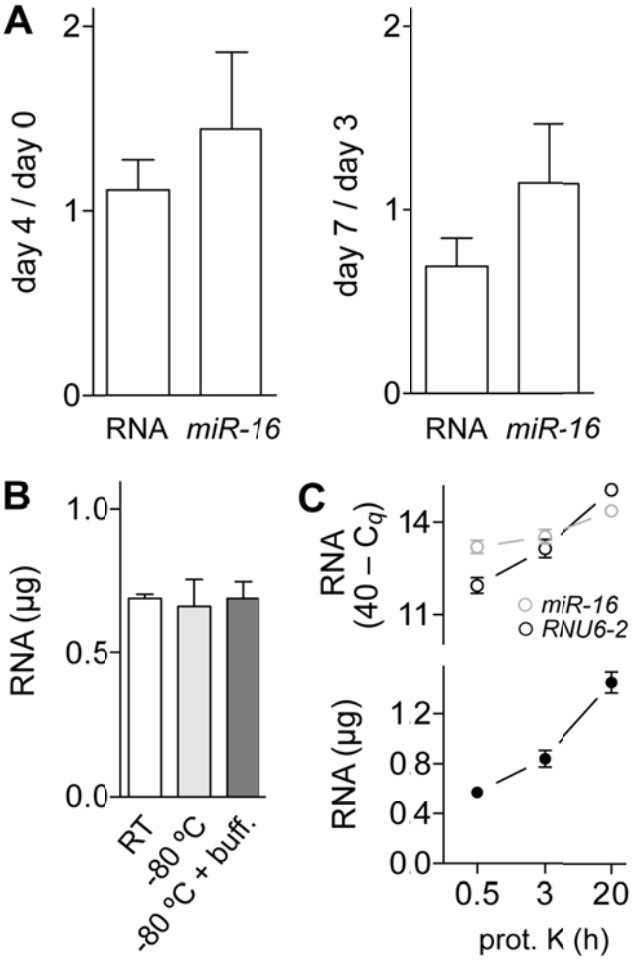
1) RNA yield is best estimated by Ribogreen assay measurements and not by absorbance at 260 nm. This conclusion was based on measurement of correlation between RNU6-2 levels and RNA quantification performed by either method in 23 dissectates (Figure 2). The Pearson correlation coefficient was 0.91 ($P<0.01$) for Ribogreen and statistically not significant ($P=0.15$) for absorbance.



2) Comparison of the use of H&E (hematoxylin and eosin) and CV (cresyl violet) demonstrated that RNA extracted after CV stain was 2-3 times higher than that with H&E. However, recognition of key histological elements was better with H &E and therefore, a decision to continue to use H & E was made (Figure 3).
3) Comparison of the use of glass fiber based columns and silica carbide based columns demonstrated 4-7 times higher yield with silica carbide columns when compared to the glass fiber columns, an impressive difference (Figure 3).
4) It is often difficult to prep slides and dissect them the same day. While this



delay decreases the quality of mRNA when frozen sections are used, such an effect may not exist with FFPE samples, such as those used in our experiments. We compared the RNA yield in samples cut on day 0, day 3 and day 7 and did not find significant differences in RNA yield. Similarly, after dissection, storage at room temperature, -80 C either in a dry state or in the tissue lysis buffer did not affect RNA yield.



5) One of the important steps involved in sample processing involves proteinase K digestion. RNA yield increases 1.5 fold when digestion time is increased from 15 min at 55 C to 3 hours. Further this yield increases by 1.7 times when the treatment time is extended from 3 to 20 hours.

6) Estimation of RNA yield by area of dissection: RNA yield from dissection can vary a great deal and it is important to have an idea of how much dissection is required to obtain a given amount of RNA. Based on the first 27 cases, we have determined that, using our protocol, an average yield of 84 ng of total RNA can be obtained from mm^2 of dissection.

As demonstrated above, a number of experiments were performed to optimize our protocol before dissection of valuable human specimens was even started. This is due to the paucity of published data and protocols that could be readily used for our project. While this has delayed our project modestly, the information gained from our protocol optimization is valuable to the scientific community in general. This data has currently been submitted for publication (Appendix 1). Our current protocol for processing of these samples is detailed

in Appendix 2.

B) **LMD of clinical samples**: So far, LMD of 57 of the proposed 77 samples has been completed. A summary of the histology of the samples, area dissected and RNA yield is presented in Appendix 3. Twenty additional samples are currently being processed. Our current work flow enables processing of 3-5 samples /wk. Therefore, this work is slated to be completed in 4-6 weeks.

Reconciliation with statement of work: According to our previously submitted statement of work, the LMD work was scheduled to be completed in the first 6 months, followed by microarray hybridization and data analysis over the next 6 months. Given the large amount of protocol optimization that was needed to be performed, we are delayed by about 3-4 months. Apart from this delay, we do not propose any alteration in the statement of work.

Key Research Accomplishments:

- Optimization of RNA yield from laser microdissectates of paraffin embedded non-small cell lung cancer specimens – these methods are useful to the scientific community engaged in this kind of research

Reportable Outcomes:

- Manuscript submission attached (Appendix 1)

Conclusion:

In the first year of this grant, we have optimized methods to greatly improve the yield of RNA from laser microdissectates of paraffin embedded non-small cell lung cancer tissues. We have also completed LMD and RNA extraction of 57 of the proposed 77 samples and are well underway to complete the first specific aim of the proposal. The methodological refinements that we have developed are of use to researchers conducting similar studies.

References:

1. Goldstraw P, Crowley J, Chansky K, et al: The IASLC Lung Cancer Staging Project: proposals for the revision of the TNM stage groupings in the forthcoming (seventh) edition of the TNM Classification of malignant tumours. J Thorac Oncol 2:706-14, 2007

2. Patnaik SK, Kannisto E, Knudsen S, et al: Evaluation of microRNA expression profiles that may predict recurrence of localized stage I non-small cell lung cancer after surgical resection. Cancer Res 70:36-45, 2010

APPENDIX

1) Manuscript: Factors affecting the yield of microRNAs from laser microdissectates of formalin-fixed tissue sections
2) LCM RNA extraction protocol summary
3) Histology, area dissected and RNA yield of samples in the first 57 samples

PLoS ONE

Factors affecting the yield of microRNAs from laser microdissectates of formalin-fixed tissue sections

--Manuscript Draft--

Manuscript Number:	
Article Type:	Research Article
Full Title:	Factors affecting the yield of microRNAs from laser microdissectates of formalin-fixed tissue sections
Short Title:	MicroRNAs from laser microdissected FFPE tissue
Corresponding Author:	Santosh Kumar Patnaik, Ph.D., M.D. Roswell Park Cancer Institute Buffalo, NY UNITED STATES
Keywords:	cresyl violet; formalin-fixed tissue; hematoxylin-eosin; laser microdissection; microRNA; RNA isolation
Abstract:	Quantification of microRNAs in specific cell populations microdissected from tissues can be used to define their biological roles, and to develop and deploy biomarker assays. In this study, a number of variables were examined for their effect on the yield of microRNAs in samples obtained from formalin-fixed paraffin-embedded tissues by laser microdissection. MicroRNA yield was improved by using cresyl violet instead of hematoxylin-eosin to stain tissue sections in preparation for microdissection, silicon carbide instead of glass fiber as matrix in RNA-binding columns, and overnight digestion of dissected samples with proteinase K. Storage of slides carrying stained tissue sections at room temperature for up to a week before microdissection, and storage of the microdissectates at room temperature for up to a day before RNA extraction did not adversely affect microRNA yield. These observations should be of value for the efficient isolation of microRNAs from microdissected formalin-fixed tissues with a flexible workflow.
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	Eric Kannisto
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Suggested Reviewers:	Patricia Meitner Rhode Island Hospital, Providence, RI, USA pmeitner@lifespan.org Dr. Meitner has worked and published on RNA isolation from FFPE tissue microdissectates.
	Xiaowei Xu Associate Professor, University of Pennsylvania School of Medicine, Philadelphia, PA, USA xug@mail.med.upenn.edu Has published on microRNA isolation from FFPE tissues
Opposed Reviewers:	

1st September 2011

To PLoS ONE

We are hereby submitting our manuscript entitled "Factors affecting the yield of small RNAs from laser microdissectates of formalin-fixed tissue sections" by Patnaik, et al., for consideration for publication in *PLoS ONE*.

To the best of our knowledge, this is the first systematic study of variables of practical importance affecting the yield of microRNAs and other small RNAs from microdissected tissue specimens. Quantification of microRNAs in such specimens is of value for biomarker discovery as well as biological studies, and we believe our work will be of interest to many.

We thank you and the journal for considering this manuscript.

Sincerely, and on behalf of all authors,

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Factors affecting the yield of microRNAs from laser microdissectates of formalin-fixed tissue sections

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Short title

MicroRNAs from laser microdissected FFPE tissue

Joint first authorship

SKP and EK contributed equally to this work

Abstract

Quantification of microRNAs in specific cell populations microdissected from tissues can be used to define their biological roles, and to develop and deploy biomarker assays. In this study, a number of variables were examined for their effect on the yield of microRNAs in samples obtained from formalin-fixed paraffin-embedded tissues by laser microdissection. MicroRNA yield was improved by using cresyl violet instead of hematoxylin-eosin to stain tissue sections in preparation for microdissection, silicon carbide instead of glass fiber as matrix in RNA-binding columns, and overnight digestion of dissected samples with proteinase K. Storage of slides carrying stained tissue sections at room temperature for up to a week before microdissection, and storage of the microdissectates at room temperature for up to a day before RNA extraction did not adversely affect microRNA yield. These observations should be of value for the efficient isolation of microRNAs from microdissected formalin-fixed tissues with a flexible workflow.

Keywords

cresyl violet; formalin-fixed tissue; hematoxylin-eosin; laser microdissection; microRNA; RNA isolation

Introduction

Laser microdissection (LMD) [1] is commonly used for the selective isolation of cell populations from tissues for molecular analyses. LMD is performed under microscopy, and cells are dissected out using a laser beam after they are identified by features such as histologic morphology. Quantification of the ultrashort, non-coding microRNAs in microdissected cells is an effective approach to understand the physiological roles of microRNAs [2,3,4,5] as well as to characterize microRNA dysregulation in diseases [6,7,8,9,10]. Unlike the much longer transcript mRNAs, microRNAs are resistant to fragmentation, and this permits the use of archived tissue material like formalin-fixed and paraffin-embedded (FFPE) specimens instead of fresh-frozen ones for reliable microRNA measurements for various studies [11,12,13]. Many of the variables that affect the recovery of microRNAs from macroscopic FFPE tissues have been identified [14,15,16,17]. However, the amount of cellular material obtained with LMD is minute, and the technique itself introduces conditions such as the presence of histologic dyes in the dissectates. In this study, we have examined some such factors of practical importance that can affect the yield and quality of microRNAs from LMD microdissectates of FFPE tissues for downstream analysis.

Materials and Methods

Ethics statement

This project was approved by the Institutional Review Board of the Roswell Park Cancer Institute (RPCI).

Tissues and microdissection

FFPE tissues of human non-small cell lung cancer and their xenografts in immunodeficient mice were kindly provided by, respectively, the core pathology facility of RPCI, and Dr. Bonnie Hylander of the Department of Immunology, RPCI. Tissue blocks were cut on a CUT4055 rotary microtome (Triangle Biomedical Sciences®, Durham, NC) into 8 µm-thick sections, which were placed on glass slides covered with a polyethylene naphthalate membrane (PEN; Leica®, Wetzlar, Germany). Slides were dried overnight, de-paraffinized with xylene and rehydrated using a graded ethanol series (100%, 99%, 75%, and 50%, by volume in water) for staining with either cresyl violet (CV; 5 mg/ml in 20% ethanol and 1.5% acetic acid at pH 2.5; Ambion®, Austin, TX), or hematoxylin and eosin (H&E) using Harris hematoxylin (Polysciences®, Warrington, PA) followed by eosin Y (5 mg/ml; Fisher Scientific®, Pittsburgh, PA) according to protocols provided by the manufacturers. Slides were then dehydrated using a reverse graded ethanol series and xylene, and used for laser microdissection within a day. LMD was performed with a pulsed ultraviolet laser on an LMD6000 system (Leica®) at 50x-200x magnification with laser power, speed and specimen-balance settings of 98, 2 and 11, respectively, in a room with >35% humidity. Dissectates were collected in 0.5 ml polypropylene tubes. The duration of LMD to obtain a dissectate sample varied from 15 to 120 minutes. Dissectates were also obtained by manually excising tissue sections along with the PEN membrane with a scalpel blade. Morphologically identical quadrants of serial sections were cut for replicate samples. All work was done with precautions to maintain an RNAse-free environment.

Isolation of RNA

Total RNA was isolated using protocols and reagents supplied with the RecoverAll™ Total Nucleic Acid Isolation (product number AM1975; Ambion®), miRCURY™ Cell and Plant Tissue RNA Isolation (product number 300110; Exiqon®, Vedbaek, Denmark), and FFPE RNA Purification (product number 25300; Norgen Biotek®, Thorold, Canada) kits. All three kits contain spin columns with an RNA-binding matrix: ~0.01 g silica or glass fiber (GF) in case of RecoverAll™, and ~0.1 g carborundum or silicon carbide (SiC) powder in the other two. The columns provided with the kits of Exiqon® and Norgen Biotek® are identical as Exiqon® procures the columns from Norgen Biotek®. Lysis of tissues and treatment with proteinase K at 55º C before a lysate was loaded on columns were done using reagents and instructions provided with the FFPE RNA Purification or the High Pure™ miRNA Isolation (product number 05 080 576 001; Roche®, Indianapolis, IN) kits. The concentration of proteinase K in the reactions set up as per the methods recommended for the two kits were 0.65 and 5.7 µg/µl respectively. Loading of lysates on a column and column washes were done using solutions and protocols supplied with the kit for that column. RNA was eluted from a column using either 50 or 100 µl water with the same volume used for all elutions in any given experiment.

Semi-quantification of RNAs by reverse transcription-PCR (RT-PCR)

TaqMan™ MicroRNA RT-PCR assay (Applied Biosystems®, Foster City, CA), with identification number 391, was used to measure microRNA *miR-16*. A similar assay was designed as per principles outlined in previous studies [18,19], validated (figure S2),

and used to quantify the small nucleolar RNA *RNU6-2* (also known as *U6B*). Sequences (and final concentrations in reactions) of the RT, and forward and reverse PCR primers, and the TaqMan™ probe were, respectively, GTCGTA TCCAGT GCAGGG TCCGAG GTATTC GCACTG GATACG ACAAAA ATAT (50 nM), GTGCAG GGTCCG AGGT (1 µM), GCAAGG ATGACA CGCAAA T (1 µM) and TATGGA ACGCTT CACGA (200 nM). For the RT-PCR assays, 5 µl each of RNA preparations were reverse transcribed using RNA-specific primers and reagents provided with the TaqMan™ MicroRNA Reverse Transcription Kit (Applied Biosystems®). RT reactions were used as templates in 40 cycle-PCR reactions on a 7900HT real-time PCR machine (Applied Biosystems®). Quantification cycle (C_q) values, approximately inversely proportional to $\log_2$ values of analyte RNA concentrations, were obtained with SDS™ software (version 2.4; Applied Biosystems®). The average of C_q values of triplicate PCR reactions was used for analysis. C_q values were >40 for negative controls, for which RT reactions were performed without RNA. C_q values were subtracted from 40 to obtain measurements directly proportional to $\log_2$ values of analyte RNA concentrations.

RNA quantification using RiboGreen assay

Nucleic acid concentration in RNA preparations was quantified in duplicate with Quant-it™ RiboGreen RNA reagent (Invitrogen®) as per the method suggested by the manufacturer. Yeast tRNA (Ambion®) was used to prepare standards of known RNA concentration. RNA samples (1-4 µl) were diluted to 100 µl using 10 mM tris hydrochloride with 1 mM ethylenediaminetetraacetic acid at pH 7.5 (CellGro®, Manassas, VA), and mixed with 100 µl of the buffer with 200- or 2000-fold diluted

RiboGreen (for high- and low-range assays, respectively). Fluorescence at 535 nm following excitation at 485 nm was measured for 0.1 s on a Victor Wallac™ 1420 plate reader (Perkin Elmer®, Waltham, MA). Unknown RNA concentrations were extrapolated from standard curves generated for yeast tRNA.

Nuclease treatment of RNA preparations

Bovine pancreas RNAse A (DNAse- and protease-free) and recombinant DNAse I (RNAse-free) were obtained from Fermentas® (Glen Burnie, MD). Ten µl of nuclease reactions were set up at 37 ºC for 1 h using 1 U of either enzyme, buffer provided by Fermentas® for use with DNAse I, and 8 µl of RNA preparation containing <0.1 µg RNA as per RiboGreen assay. Control reactions using yeast tRNA (0.1-0.2 µg) confirmed completeness of the RNAse reactions and absence of RNAse activity in the DNAse I stock.

MicroRNA profiling using locked nucleic acid (LNA) microarrays

This work was performed as a commercial service by Exiqon® (Vedbaek, Denmark) using their 6th generation miRCURY™ LNA™ microarrays. Each array had more than 2383 LNA capture probes for multiple RNAs of human, mouse, rat, and some viruses printed in quadruplicate on randomly distributed spots of 105 µm diameter with an inter-spot distance of 210 µm. A total of 1304 probes targeted 1291 human microRNAs, including 66 proprietary ones (miRPlus™, Exiqon®), and 23 non-microRNA human small RNAs with <200 nucleotides, including the *5S* ribosomal RNA and the *RNU6-2* small nucleolar RNA (*U6B*). Every microRNA was recognized by only one of the 1276

probes for microRNAs. Eight probes recognized two microRNAs each, and three and six microRNAs were recognized by one probe each. For simplicity, the signals from such probes were considered as representing single microRNAs. Before hybridization to a microarray, 0.25 or 0.4 μg of an RNA sample, reduced in volume at room temperature in a speed-vacuum apparatus, and a human 'universal reference' total RNA preparation made by mixing the RNA pools provided in the FirstChoice® Human Total RNA Survey Panel (product number AM6000, Ambion®, Austin, TX) were 3'- or 5'-end-labeled with Cy3-like Hy3™ or Cy5-like Hy5™ (Exiqon®) dyes, respectively, using miRCURY™ LNA™ microRNA Hi-Power Labeling kits (Exiqon®). Microarrays were scanned for analysis using ImaGene® software (version 9; BioDiscovery®, Los Angeles, CA). Examinations of the scans and analyses of microarray signal values for 52 spiked-in synthetic, small RNAs showed that all labeling reactions and hybridizations were of good quality. Hy3™ and Hy5™ signal values were processed with the limma [20] Bioconductor package (version 3.6.9) for R (version 2.12). Correction for background noise was done using the normexp method [21] with an 'offset' value of 10, and was followed by within-array normalization using the global loess regression method with a 'span' value of 1/3 [22]. Microarray signal values were then identified as summarized Hy3™ values which were the means of values from the quadruplicate probe-spots when the maximum was <1.5 times the minimum, or the medians if otherwise. MicroRNAs recognized by probes for which the microarray signal values were >3 times the summarized microarray signal value for probe-less empty microarray spots (1108 total) were considered as expressed.

Other

Unless specified otherwise, statistical analyses and graphical plotting were done in Prism™ software (version 5.0d; GraphPad Software®, La Jolla, CA), P value of 0.05 was the cut-off for deciding significance, and t tests were two-tailed, assumed equal variances, and used paired samples when possible.

Results and Discussion

We obtained FFPE tissues of human lung cancers or their xenografts grown in mice for this work. Tissues were cut into 8 µm-thick sections, which were then placed on glass slides covered with PEN membrane. The sections were deparaffinized and stained with either H&E or CV, and used for LMD within a day with a pulsed ultraviolet laser on an LMD6000 system (Leica®). For some experiments, areas of tissue sections were dissected out along with PEN membrane by hand using a surgical blade. To obtain replicate samples, morphologically identical quadrants of stained serial sections were cut. Dissectates were lysed with proteinase K and total RNA was extracted by affinity chromatography using the RecoverAll™ Total Nucleic Acid Isolation (Ambion®) or FFPE RNA Purification (Norgen Biotek®) kits that respectively use GF or SiC as the RNA-binding matrix. Total RNA in RNA extractions was quantified using RiboGreen dye in a fluorescence assay [23], or by measuring absorbance at 260 nm. Identical volumes of different RNA preparations were used for TaqMan™ microRNA assays (Applied Biosystems®), based on RT-PCR [18], for microRNA *miR-16*, an abundant and ubiquitous microRNA [e.g., 24], and *RNU6-2* (*U6B*), a 45 base-long, housekeeping nucleolar RNA. Inter-group differences were analyzed using t tests assuming equal

variances. P values determined in different statistical tests were two-tailed and a cut-off of 0.05 was used to appraise significance.

An analysis of RNA preparations from 23 different dissectates from xenografts showed that *RNU6-2* levels correlated well with total RNA estimations by RiboGreen assay with a Pearson coefficient of 0.91 (95% confidence interval = 0.79-0.96; $P < 0.01$) whereas there was no significant correlation with total RNA quantifications by absorbance at 260 nm ($P = 0.15$; figure 1). RiboGreen assay was thus deemed as more precise than absorbance spectrophotometry for RNA samples of low concentration, as has been observed by others [25], and was used to assess total RNA for the rest of the study. H&E and CV are nucleic acid-binding stains that can possibly interfere with RNA extraction, and their use can differentially affect RNA degradation during the processing steps of staining [26,27,28]. To evaluate this, we compared small RNA yields from H&E- or CV-stained replicate dissectates from three xenografts by measuring *RNU6-2* and *miR-16* levels. In RNA extracted using GF columns, *RNU6-2* and *miR-16* levels respectively were an average of 2.1 and 3.0 times higher with CV than H&E (figure 2A). With SiC columns too, *RNU6-2* and *miR-16* levels were, respectively, on average 2.6 and 2.0 times higher with CV than H&E. In paired t tests disregarding the column-type, the improvements in *RNU6-2* and *miR-16* yields were significant (P values of 0.02 and 0.01, respectively). Because of convenience, we decided to use H&E stain for the rest of the experiments of this study. The efficacies of the two types of columns were also compared. For this, proteinase K lysates were prepared from dissectates from three xenografts and divided into two equal portions, each of which was used for the two types of columns. As shown in figure 2B, with CV-stained dissectates, *RNU6-2* and

miR-16 levels respectively were an average of 3.9 and 7.0 times higher with SiC columns than GF columns. When H&E was the stain, *RNU6-2* and *miR-16* levels were, respectively, on average 3.7 and 7.9 times higher with SiC columns than GF columns. These improvements in *RNU6-2* and *miR-16* yields, significant in paired t tests disregarding the histologic stain (both P values <0.01), could be because of differences in column design and not necessarily because of a better efficacy of the SiC matrix per se.

To test effect on RNA yield of duration of storage of stained slides at room temperature before dissection and RNA extraction, replicate sections from three xenografts were used for dissection on the same day (day 0) the slides were prepared or after a period of 3-7 days. RiboGreen and *miR-16* assays of the RNA preparations showed that RNA yields were not reduced at day 4 compared to day 0, or at day 7 compared to day 3 (figure 3A). This observation indicates that slides can be prepared and stored for at least a week before LMD is performed without an adverse effect on microRNA yield. The effect of different storage conditions for dissectates before RNA extraction was also examined (figure 3B). There was no significant difference in RNA yield as measured by RiboGreen assay between LMD samples kept at room temperature for a day in a dry state, or at –80 ºC either in a dry state or in the tissue lysis buffer provided with the RNA extraction kit.

As expected from previous studies on RNA extraction from FFPE tissues [e.g., 16], RNA yield improved significantly when the duration of proteinase K treatment was extended (figure 3C). RiboGreen, *RNU6-2* and *miR-16* measurements respectively were on average 1.5, 2.3 and 1.3 times higher when the duration was increased to 3 h

at 55 ºC from 15 min at 55 ºC followed by 15 min at 80 ºC (P values of 0.02, 0.04 and 0.36, respectively). Extending treatment time from 3 to 20 h resulted in 1.7, 3.8 and 1.8 times higher RiboGreen, *RNU6-2* and *miR-16* measurements, respectively (P values of <0.01, <0.01 and 0.03, respectively).

To assess the relation of dissectate quantity and RNA yield, epithelial compartments of 27 human non-small cell lung cancers were isolated by LMD from H&E-stained FFPE tissue sections, and digested with proteinase K at 55 ºC overnight. RNA from the lysates was prepared using the kit from Norgen Biotek®. As shown in figure 4A, there was a significant Pearson correlation (r = 0.71, 95% confidence interval = 0.45-0.86) between cross-sectional areas of dissectates and RiboGreen measurements of RNA prepared from them, with an average of 84 ng RNA obtained per mm^2 area. RiboGreen assay of four different RNA preparations that were treated with DNAse I, RNAse A or neither at 37 ºC for 1 h showed that 37%-39% of the nucleic acids in the RNA preparations was DNA and not RNA (figure 4B). To assess the suitability of the RNA for microRNA quantification using microarrays, 250 or 400 ng of one RNA sample was labeled with Hy3™ dye and hybridized in duplicate to miRCURY™ locked nucleic acid microarrays (Exiqon®). With both 250 and 400 ng input, about 56% of the 1291 microRNAs detectable by the microarrays were identified as expressed. However, microarray signals were stronger with higher RNA input (figure 4C). E.g., 21% of expressed microRNAs had signal values of >200 for with 400 ng RNA whereas the value was 17% for 250 ng. Inter-duplicate correlation analyses showed that microarray signals were likely more accurate and less noisy when more RNA was used (figure 4C). Comparison of microarray signal from RNA prepared from microdissectates with that

from the commercially available human 'universal reference' RNA, which was used for the reference channel of the two-color microarrays, showed that the microRNA isolation method did not adversely affect RNA labeling and hybridization for microarray analysis (figure S2).

To summarize, this study suggests that microRNA yields from LMD samples obtained from FFPE tissues can be improved by using CV instead of H&E as histologic stain, SiC instead of GF as matrix in RNA-binding columns, and overnight digestion with proteinase K. Storage of stained slides at room temperature for up to a week before LMD, and storage of LMD samples at room temperature for up to a day before RNA extraction does not seem to adversely affect microRNA yield. RNA prepared as per the methods used in this study, though containing DNA as well, appear to be suitable for microRNA quantification by RT-PCR or microarray hybridization. These observations should allow for efficient isolation of microRNAs from microdissectates prepared from FFPE tissues with a more manageable and flexible workflow.

Acknowledgements

We thank Wiam Bshara, Zahra Fayazi, Angela Omilian and Melanie Kresin of the Department of Pathology, RPCI for slide preparation and assistance with LMD.

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Figure Legends

Figure 1. Scatter-plots of RNA concentration and RNU6-2 measurements of RNA from dissectates of formalin-fixed tissue sections

Total RNA in 23 samples was quantified by RiboGreen assay (*black*) or absorbance spectrophotometry at 260 nm (*grey*). Level of *RNU6-2* in the RNA preparations was determined as quantification cycle (C_q) values obtained in reverse transcription-PCR assays.The best lines of fit with the least squares method are also shown.

Figure 2. Effect of histologic stain and RNA-binding matrix in spin-columns on RNA yield

Yields with cresyl violet (*CV)* stain relative to hematoxylin and eosin (*H&E*) for glass fiber (*GF*) and silicon carbide (*SiC*) columns (*A*), and with SiC relative to GF columns for both stains (*B*) are plotted as means with their standard errors for dissectates from three tissues. Log_2-transformed *RNU6-2* and *miR-16* levels were determined from C_q values obtained in reverse transcription-PCR assays.

Figure 3. Effect of age of slides and dissectates, and proteinase K treatment duration on RNA yield

A. Total RNA and *miR-16* yields from laser microdissectates from three tissues prepared from four or seven day-old slides relative to zero or three day-old ones, respectively. *B*. Total RNA yield from identical laser dissectates from zero day-old slides stored in duplicate at room temperature (*RT*), or at -80 ºC with or without buffer (*buff.*) for a day. *C*. Total RNA yield (*filled circles*) and levels of *RNU6-2* (*black empty circles*) and *miR-16* (*grey empty circles*) from identical dissectates treated in triplicate with

proteinase K (*prot. K*) for 0.5, 3 or 20 h. Means and their standard errors are plotted. Log_2-transformed *RNU6-2* and *miR-16* levels were determined from quantification cycle (C_q) values obtained in reverse transcription-PCR assays. Total RNA was quantified by RiboGreen assay. Hematoxylin-eosin was used as the histologic stain, and silicon carbide columns were used for RNA isolation.

Figure 4. Assessment of RNA prepared from FFPE tissue microdissectates

A. Scatter-plot of area and RNA yield as per RiboGreen assay for 27 tissue samples obtained by laser microdissection (LMD). The best line of fit with the least squares method is shown. *B*. Measurements in RiboGreen assay following treatment of four RNA preparations with RNAse A or DNAse I enzyme relative to treatment without either. Means with their standard errors are shown. *C*. Microarray signal values (*dots*) and inter-duplicate Pearson correlation coefficient, r (*lines*) for 747 microRNAs measured in duplicate using 250 (*grey*) or 400 ng (*black*) of RNA prepared from an LMD sample. A rolling window of width 99 along the X axis was used for calculating value of r at the mid-window abscissa.

Supporting Information Legends

Figure S1

Validation of a custom reverse transcription (RT)-PCR assay for RNU6-2. *A*. Quantification cycle (C_q) values were determined for 40, 15 or 5 ng total RNA isolated from cells derived from the A549 human lung cancer cell-line. The TaqMan™ microRNA RT-PCR assay with ID 1093 from Applied Biosystems® (*ABI*) or a similar but custom

assay for the *RNU6-2* nucleolar RNA were used. The two assays were different for only the primers and probes. The linear regression line (ordinary least squares method) for the scatter-plot is also shown. The Pearson correlation coefficient is >0.99 (P = 0.02). *B*. An ethidium bromide-stained agarose gel (2%) after electrophoresis of the RT-PCR products for the assays with 40 ng RNA input was transilluminated with ultraviolet light and photographed. Sizes of DNA molecular weight markers (Invitrogen®, Carlsbad, CA) in base-pairs (*bp*) are shown. The RT-PCR product expected in the custom assay has a size of 75 bp.

Figure S2

Labeling of RNA prepared from dissectates and hybridization to microarrays. Two-hundred-fifty or 400 ng each of a human 'universal reference' total RNA (Ambion®) and RNA prepared from laser microdissected tissue using FFPE RNA Purification kit (Norgen Biotek®) were respectively labeled with the Hy5™ and Hy3™ dyes, and co-hybridized to a locked nucleic acid microarray (Exiqon®). Fifty-two different synthetic artificial microRNAs were exogenously added to the RNAs before labeling. Scatter-plots of the Hy5™ and Hy3™ microarray signal values for the 52 spike-ins, and their linear regression lines (ordinary least squares method) are shown. The slopes of the lines are 0.70 and 0.81 for 250 and 400 ng RNA input, respectively, suggesting that the method used to isolate RNA from dissectates did not negatively affect the labeling and hybridization of the RNA.

Figure 1

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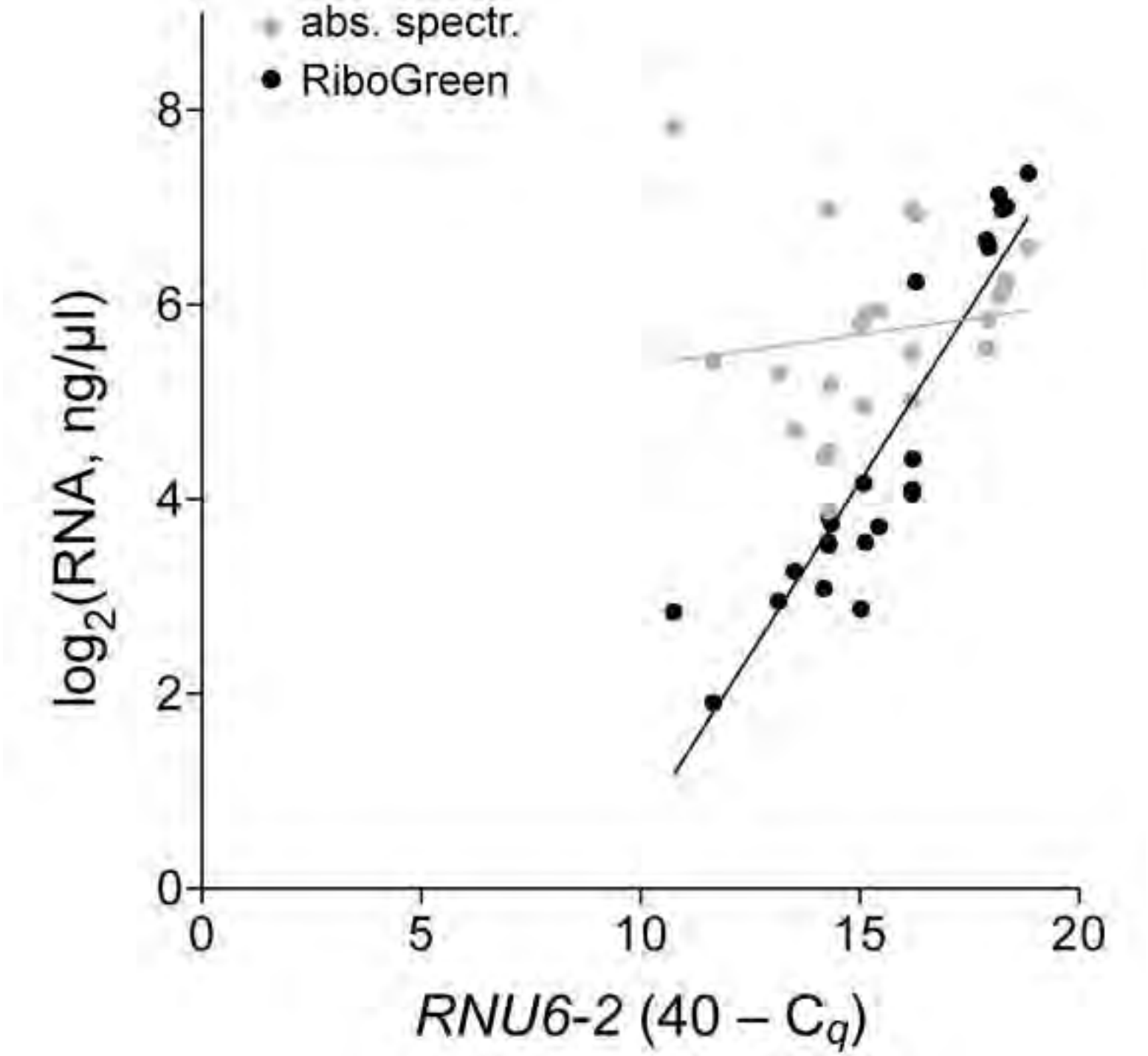

Figure 2

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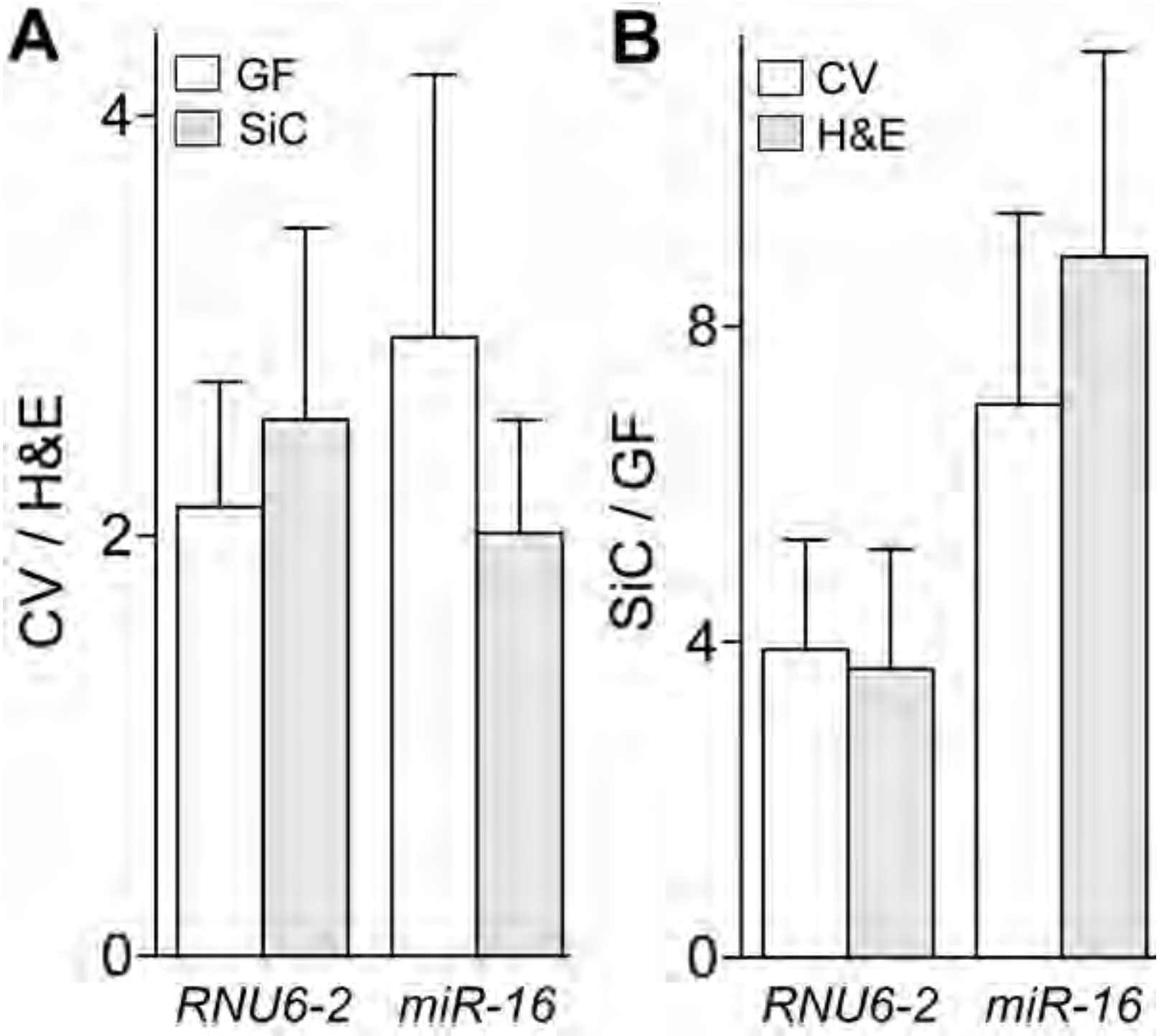

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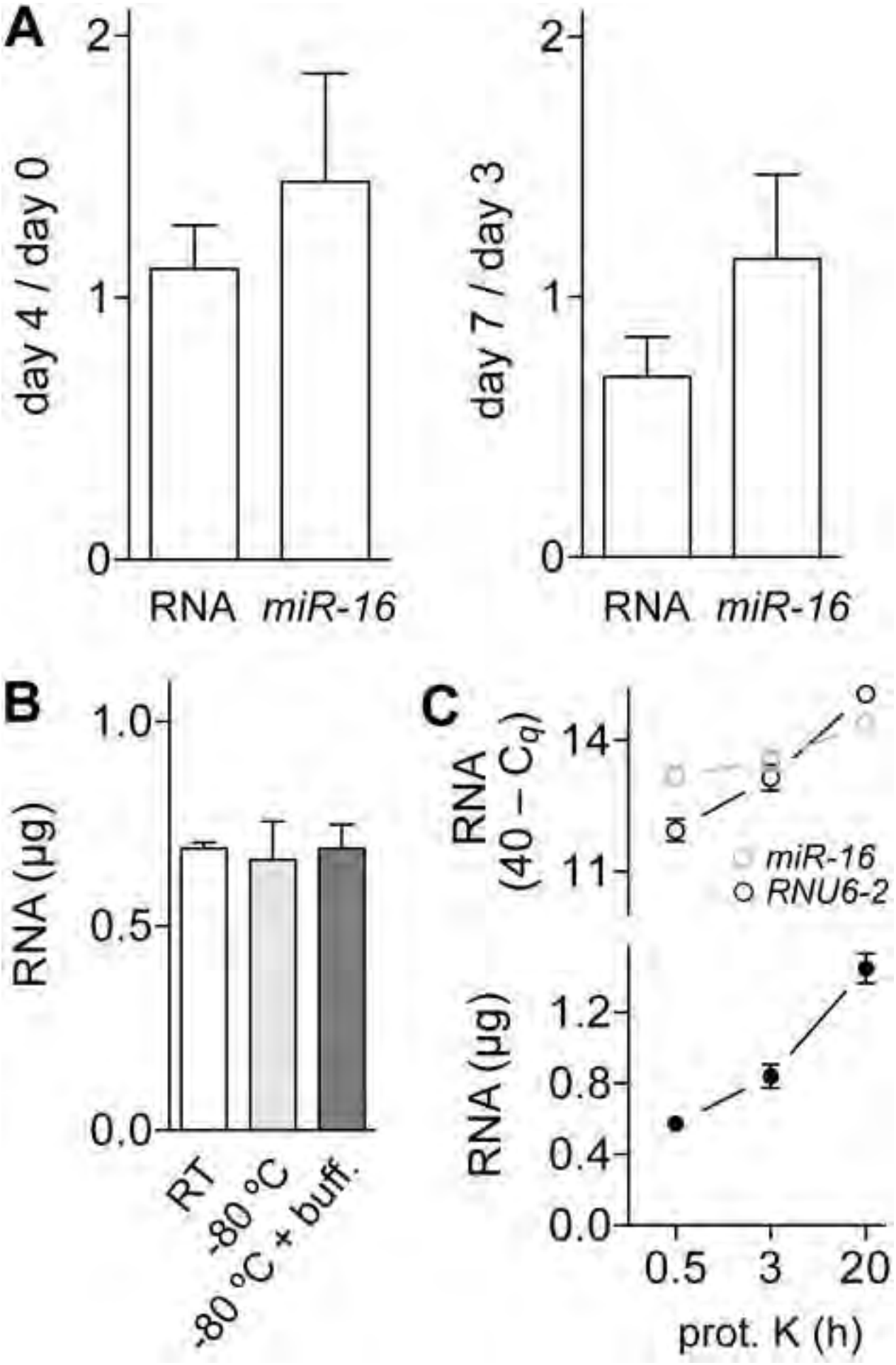

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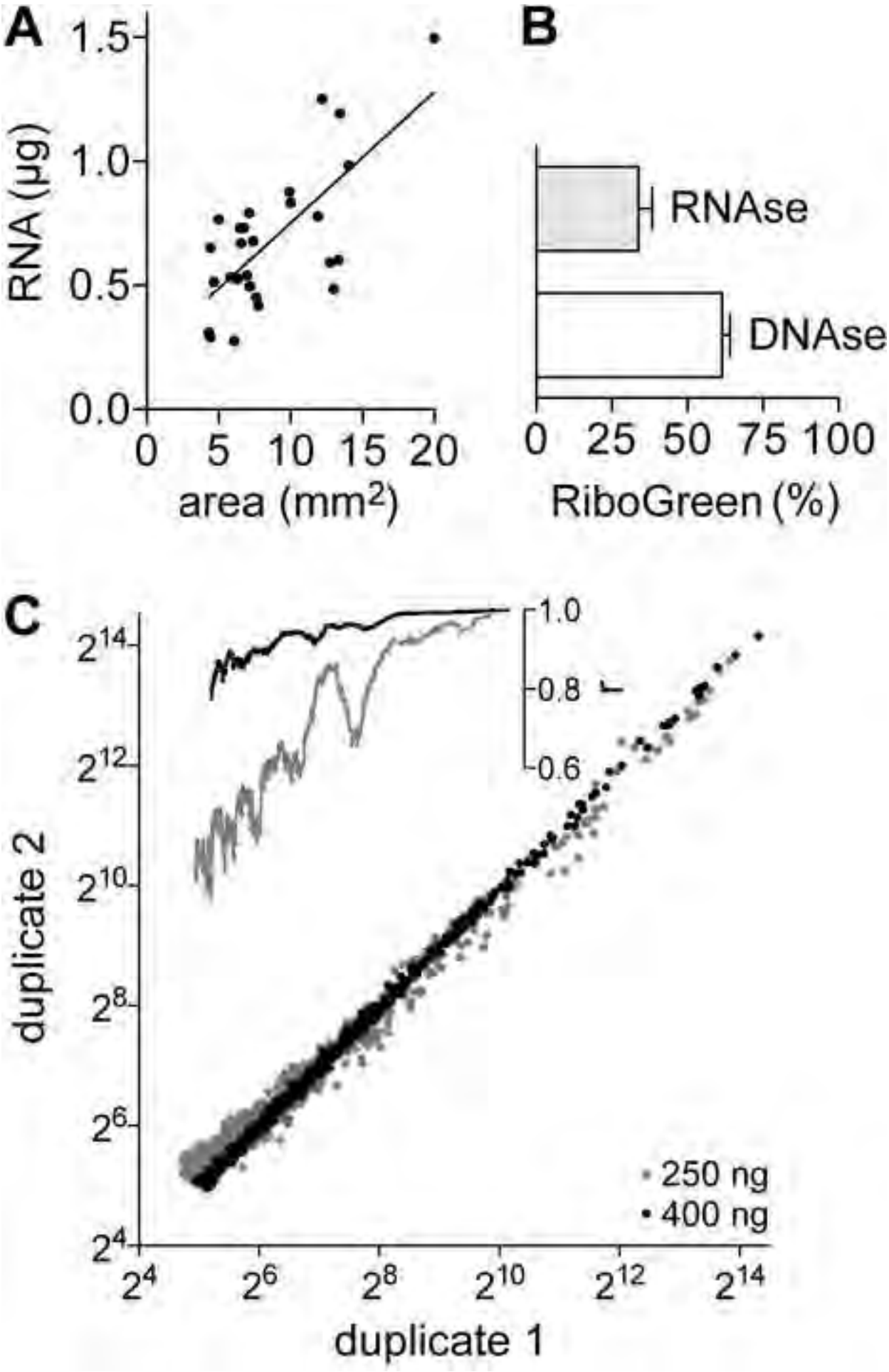

Figure S1

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Figure S2

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APPENDIX 2

LCM RNA extraction protocol summary

LCM slides were prepared with 8µm tissue sections from FFPE blocks and stored covered at room temperature. After staining with hematoxylin and eosin, slides were cut within a week of being prepared on a Leica LMD6000 with settings of laser power: 98, laser speed: 2 at magnifications ranging from 5-20x. Epithelial and stroma sections were collected in separate tubes from 3-6 slides for each sample. Once recovered, the dissected tissue was stored in 300µL digestion buffer (Norgen® FFPE RNA purification kit) at -80ºC for up to 1 week before being extracted. For RNA extraction, samples were thawed at room temperature and 10µL of proteinase K (supplied by manufacturer) was added. Samples were vortexed to mix and stored at 55°C overnight. The following day RNA was extracted using FFPE RNA purification kit (Norgen®), eluted in 100µL nuclease-free water according to manufacturer protocol on a QIAvac™ (Qiagen®) and stored at -80°C.

RNA was quantified using Quant-It™ Ribogreen® RNA reagent (Invitrogen™) according to manufacturer protocol. Briefly, RNA samples were diluted 1:200 in 1X TE buffer, pH 8.0 (CellGro®) and 100µL is added to duplicate wells of a 96 well plate. Standards were made using yeast tRNA (Ambion®) to known concentrations in 1X TE buffer and 100µL was added to duplicate wells on the same 96 well plate. Ribogreen reagent was diluted 1:2000 in 1X TE buffer and 100µL was mixed with standards and RNA samples. Samples were excited at 485nm and fluorescence was measured at 535nm for 0.1 seconds on a Victor Wallac™ 1420 plate reader (Perkin Elmer®).

Appendix 3. Histology, area dissected and RNA yield of samples in the first 57 samples. Samples without RNA yield information have not had the RNA quantified.

Sample	Type	Tissue	Total area (μm^2)	ng RNA (in 100µL DDW)
E1	SCC	Epithelia	11,889,137	778.2
S1		Stroma	10,195,533	606.6
E2	SCC	Epithelia	6,336,667	526.1
S2		Stroma	8,172,244	664.3
E3	SCC	Epithelia	6,970,549	540.0
S3		Stroma	3,067,083	436.4
E4	AC	Epithelia	6,489,975	732.5
S4		Stroma	6,832,705	468.6
E5	AC (mucinous)	Epithelia	3,585,262	462.9
E6	SCC	Epithelia	7,386,451	679.2
S6		Stroma	14,173,228	553.1
E7	SCC	Epithelia	4,436,510	651.3
S7		Stroma	5,531,488	527.5
E8	SCC	Epithelia	6,564,047	669.1
S8		Stroma	7,499,256	512.4
E9	Bronchiolo-alveolar carcinoma	Epithelia	6,756,473	731.0
S9		Stroma	7,933,855	539.1
E10	AC	Epithelia	8,921,590	410.7
S10		Stroma	13,135,022	107.1
E10a		Epithelia	7,613,816	451.7
S10a		Stroma	15,834,363	433.8
E11	SCC	Epithelia	4,660,802	513.8
S11		Stroma	3,717,275	260.7
E11a		Epithelia	9,507,825	
S11a		Stroma	4,877,403	
E12	AC	Epithelia	6,089,080	276.7
S12		Stroma	5,864,063	484.7
E12a		Epithelia	2,438,439	466.6
S12a		Stroma	2,475,640	471.1
E13	AC	Epithelia	7,121,877	791.2
S13		Stroma	5,613,407	614.1
E14	AC	Epithelia	10,014,712	833.3
S14		Stroma	5,452,384	633.8
E15	AC	Epithelia	7,158,702	496.1
S15		Stroma	3,324,505	401.5
E16	Bronchiolo alveolar adenocarcinoma	Epithelia	5,823,184	533.1
S16		Stroma	6,949,284	510.8
E17	SCC	Epithelia	12,212,901	**1251.0**
S17		Stroma	9,327,528	661.8
E18	SCC	Epithelia	33,568,594	1207.3
S18		Stroma	11,881,325	520.1
E19	SCC	Epithelia	7,776,541	418.2
S19		Stroma	5,134,259	392.4
E19a		Epithelia	26,587,354	
S19a		Stroma	9,863,967	
E20	AC	Epithelia	19,989,848	**1496.5**
S20		Stroma	14,165,658	604.2
E21	AC	Epithelia	36,214,523	**2466.3**

Appendix 3. Histology, area dissected and RNA yield of samples in the first 57 samples. Samples without RNA yield information have not had the RNA quantified.

Sample	Type	Tissue	Total area (μm^2)	ng RNA (in 100μL DDW)
S21		Stroma	17,993,940	575.1
E22	AC	Epithelia	12,724,641	591.9
S22		Stroma	10,205,653	541.2
E23	Bronchiolo alveolar adenocarcinoma	Epithelia	13,338,701	602.9
S23		Stroma	10,555,984	348.2
E23a		Epithelia	6,982,869	605.6
S23a		Stroma	4,855,874	545.9
E24	SCC	Epithelia	33,419,779	1066.4
S24		Stroma	20,235,708	745.9
E25	Bronchiolo alveolar adenocarcinoma	Epithelia	4,304,297	309.8
S25		Stroma	3,247,737	334.3
E25a		Epithelia	2,483,175	
S25a		Stroma	2,199,418	
E26	AC (mucin producing)	Epithelia	4,417,796	292.3
S26		Stroma	2,523,781	265.1
E26a		Epithelia	13,945,809	
S26a		Stroma	4,433,235	
E27	SCC	Epithelia	48,980,212	**3665.6**
S27		Stroma	27,215,417	**1251.2**
E28	SCC	Epithelia	29,368,036	**1490.3**
S28		Stroma	17,170,347	1037.3
E29	SCC	Epithelia	13,422,037	**1192.4**
S29		Stroma	12,759,225	726.8
E30	AC	Epithelia	9,904,198	878.0
S30		Stroma	3,800,906	410.0
E31	Bronchiolo alveolar adenocarcinoma	Epithelia	14,047,871	983.6
S31		Stroma	6,634,291	444.4
E32	AC	Epithelia	13,012,456	485.2
S32		Stroma	14,393,574	569.1
E33	AC	Epithelia	~5,000,000	766.0
S33		Stroma	4,643,523	406.6
E34	SCC	Epithelia	4,958,524	708.8
S34		Stroma	3,553,901	325.4
E34a		Epithelia	4,985,359	682.3
S34a		Stroma	2,566,021	421.4
E35	AC	Epithelia	10,950,192	632.2
S35		Stroma	9,603,913	460.0
E36	AC	Epithelia	5,404,904	502.9
S36		Stroma	10,074,331	709.9
E37	SCC	Epithelia	6,645,826	704.2
S37		Stroma	8,934,385	572.0
E38	AC	Epithelia	6,874,579	562.8
S38		Stroma	8,474,903	519.3
E39	SCC	Epithelia	10,176,394	1145.8
S39		Stroma	11,100,795	931.9
E40	Bronchiolo alveolar adenocarcinoma	Epithelia	12,153,127	900.7
S40		Stroma	5,769,434	406.7
E41	AC	Epithelia	3,357,060	480.6

Appendix 3. Histology, area dissected and RNA yield of samples in the first 57 samples. Samples without RNA yield information have not had the RNA quantified.

Sample	Type	Tissue	Total area (μm^2)	ng RNA (in 100µL DDW)
S41		Stroma	4,885,662	506.2
E42	Bronchiolo alveolar adenocarcinoma	Epithelia	10,500,000	646.6
S42		Stroma	5,378,919	476.3
E43	AC	Epithelia	3,687,867	345.7
S43		Stroma	4,007,605	442.2
E43a		Epithelia	3,695,170	593.7
S43a		Stroma	3,909,939	525.4
E44	AC with mixed subtypes	Epithelia	10,109,261	1176.1
S44		Stroma	5,620,523	446.0
E45	AC	Epithelia	6,000,000	**1342.9**
S45		Stroma	2,500,000	486.7
E46	AC	Epithelia	7,342,487	692.9
S46		Stroma	6,501,621	419.8
E47	AC	Epithelia	3,105,778	454.4
S47		Stroma	3,115,846	389.7
E48	Bronchiolo alveolar adenocarcinoma	Epithelia	8,844,916	726.5
S48		Stroma	6,632,574	728.8
E49	SCC	Epithelia	9,606,216	**1439.2**
S49		Stroma	5,591,155	542.2
E50	Bronchiolo alveolar adenocarcinoma	Epithelia	7,483,638	525.6
S50		Stroma	4,300,734	414.8
E51	Bronchiolo alveolar adenocarcinoma	Epithelia	6,361,522	596.0
S51		Stroma	6,905,457	430.4
E52	Bronchiolo alveolar adenocarcinoma	Epithelia	9,963,150	492.7
S52		Stroma	6,140,914	560.6
E53		Epithelia	5,585,836	660.2
S53		Stroma	5,763,095	556.9
E54	SCC	Epithelia	9,395,108	1180.6
S54		Stroma	5,495,302	629.5
E55	SCC	Epithelia	9,846,328	1153.8
S55		Stroma	4,951,730	462.2
E56	Bronchiolo alveolar adenocarcinoma	Epithelia	5,033,958	567.5
S56		Stroma	3,074,020	370.4
E56a		Epithelia	3,883,261	
S56a		Stroma	2,326,786	
E57	SCC	Epithelia	3,724,888	475.9
S57		Stroma	3,952,248	504.3
E58	AC	Epithelia	3,985,825	522.3
S58		Stroma	3,732,852	520.7